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Dynamic Protein-Phospholipid Interaction in Rhodopsin Recombinant Membranes: Rotational Motion of Rhodopsin and Phospholipid Alkyl Chain Flexibility( Dissertation\_全文 )

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**Dynamic Protein-Phospholipid Interaction in Rhodopsin  
Recombinant Membranes: Rotational Motion of Rhodopsin and  
Phospholipid Alkyl Chain Flexibility**

**Akihiro Kusumi**

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Abbreviations used are: anti DNP antisera, anti dinitro-phenylated-BSA antisera produced in goat; BSA, bovine serum albumin; DEPC, dielaidoylphosphatidylcholine; DMPC, dimyristoyl-phosphatidylcholine; DMR, deuterium magnetic resonance; DTAC, dodecyltrimethylammonium chloride; EDTA, ethylenediaminetetra-acetic acid; EPR, electron paramagnetic resonance; EYPC, egg yolk phosphatidylcholine; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MSL(maleimide spin label), N-(1-oxy-2,2,6,6-tetramethyl-4-piperidiny)-maleimide; 5- and 16-nitroxide stearate, N-oxy-4',4'-dimethyloxazolidine derivative of 5- and 16-ketostearic acid, respectively; ROS, rod outer segment; SB, standard buffer containing 10 mM HEPES and 65 mM NaCl, pH 6.8; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxy; Tempol, 2,2,6,6-tetramethylpiperidine-1-oxy.

## Abstract

Dynamical aspect of protein-lipid interaction in membranes was studied. For the purpose of investigating fundamental properties of protein-lipid interaction, it is desirable to utilize simplified materials, recombined membranes with purified protein and well defined phospholipids. Rhodopsin, a visual receptor protein in rod outer segment membrane, was chosen as a membrane protein and dimyristoylphosphatidylcholine (DMPC) or dielaidoylphosphatidylcholine (DEPC) was used as a two dimensional matrix. DEPC has longer alkyl chains (4 methylenes) than DMPC and a double bond (9-trans).

Lipid-rich recombinants with a rhodopsin/phospholipid ratio of 1/300 were prepared as a first step with DMPC and DEPC, which were examined to study how the differences in phospholipid structure affected protein-lipid interaction. Next, recombinants with various rhodopsin/DMPC ratios were investigated.

The aim of this work was to elucidate the correlation between the rotational mobility of membrane proteins and the flexibility of lipid alkyl chains in recombinants.

The rotational motion of rhodopsin and mobility of lipid alkyl chain were monitored by spin labeling method. Behaviors of hydrophobic chains of phospholipids have been studied extensively by a

variety of methods, but few works had been reported about the mobility of membrane proteins, one of the reasons of which was the lack of suitable techniques to detect a slow rotational motion with correlation times ( $\tau_2$ ) longer than microsecond. Passage saturation transfer EPR technique, developed in 1972 by Hyde and Dalton, paved the way for studies of slowly tumbling nitroxide radical with correlation times between  $10^{-7}$  s and  $10^{-3}$  s. The rotational mobility of rhodopsin was estimated by this technique applied to spin-labeled rhodopsin. The greatest trouble in application of saturation transfer spectroscopy lay in employment of identical effective  $H_1$ -field, which was deeply affected by the dielectric constant of samples, under different experimental conditions. Incident microwave powers were corrected to obtain the identical  $H_1$ -field on the sample, which had usually been overlooked. It was shown that neglect of power correction caused from two to ten-fold value in estimation of  $\tau_2$ .

Rhodopsin in frog rod outer segment membranes was shown to possess large mobility with  $\tau_2$  of around  $10^{-5}$  s, in general agreement with the transient photodichroic data by Cone. The anisotropic nature of rotational motion of rhodopsin was also demonstrated.

Behaviors of phospholipid hydrocarbon chains were observed with nitroxide-stearate spin probes by conventional EPR. Saturation

transfer EPR was also used when necessary.

Attention has been paid to the correlation between mobilities of rhodopsin and lipid alkyl chains, especially to the effect of rhodopsin on the lipid phase transition and the reflected influence of rhodopsin on the rotational motion of rhodopsin itself.

The results obtained are summarized below.

R1) Incorporated rhodopsin slowed down the alkyl chain mobility. This effect was most pronounced around the lipid phase transition, and large in the fluid state, but small in the solid state.

R2) Rhodopsin broadened the lipid phase transition to higher temperature side. This influence increased as rhodopsin/DMPC ratio increased.

R3) Rhodopsin gave larger effect on DMPC than DEPC in the fluid state.

R4) Conventional EPR spectra of 16-nitroxide stearate showed a superposition of two kinds of spectra when rhodopsin/DMPC ratio was high. Recombinant membranes with lipids just enough to cover the protein hydrophobic surface also showed two spectral components, indicating two environments around the spin label.

R5)  $\tau_2$  value estimated by means of saturation transfer EPR spectroscopy well approximated the rotational mobility of rhodopsin.

R6) The temperature profile of  $\tau_2$  of rhodopsin showed



inflexions corresponding to the phase transition of lipid moiety.

The temperature characteristics of  $\tau_z$  resembled that observed for the lipid flexibility.

R7) Increased concentration of rhodopsin in the membrane decreased the rotational mobility of the protein in the fluid state.

R8) Rhodopsin maintained some mobility in the solid state of lipid.

R9)  $\tau_z$  of rhodopsin rotation followed <sup>an</sup> identical temperature profile in every recombinant in the solid state.

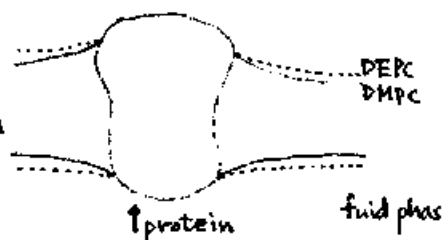
Following conclusions were obtained from the results above in conjunction with data presented by others.

C1) The influence of membrane protein on phospholipids <sup>would</sup> possibly propagate to longer distances around the lipid phase transition (R1).

C2) Membrane proteins caused the alkyl chain motion slower but larger in amplitude (R1, Kang et al.).

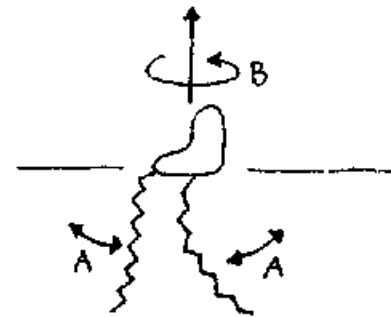
C3) Relative dimension of the protein hydrophobic surface to the fluid lipid chain length would be involved for determination of the type and range of the propagating effect of protein (see the figure above)(R3).

C4) Membrane proteins would contribute to slowing down of the motion like A mode (see the upper figure on the next page)

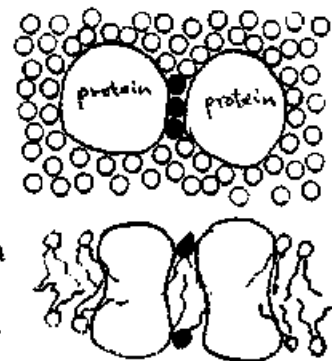


more effectively than to that of the motion of B mode (R1, R2, Kang et al.).

C5) Rhodopsin formed large clusters in the solid state (Chen and Hubbell), which reduced the effect of rhodopsin on the mobility of phospholipids (R1) and abolished the dependence of  $\tau_2$  of rhodopsin on rhodopsin/DMPC ratio (R9). The rotational mobility of rhodopsin in the solid state (R8) would reflect the motion in the cluster in the presence of protein-protein interaction.



C6) The importance of interactions between proteins was suggested (R4, C5). Protein-protein interaction would be manifested explicitly in the circumstances shown in the right figure, even if no direct contact between proteins took place.



● : lipid in contact with two proteins

## Introduction

The dynamic nature of biological membranes is one of the most essential properties that characterize <sup>their</sup> structure. Proteins and phospholipids, the major constituents of biological membranes, are interacting each other in the structure to determine their mobility. The incorporation of proteins to lipid bilayer affected the lipid alkyl chain flexibility (Griffith and Jost, 1978; Chapman et al., 1979; Favre et al., 1979; Kang et al., 1979; Kusumi et al., 1979), while the state of lipids influenced the mobility of membrane proteins (Kusumi et al., 1979).

Dynamic protein-lipid interaction has been studied mostly from the side of lipids influenced by proteins and little information has been obtained on the side of proteins affected by lipids. This has been due to lack of experimental methods to measure protein mobility in membranes, which should have correlation times as long as from microsecond to millisecond, in contrast to a variety of techniques available for lipids. Recently, several techniques have been developed for the protein lateral and rotational mobilities: recovery after photo-bleaching <sup>(of)</sup> fluorescence (Edidin et al., 1976; Koppel et al., 1976), transient photodichroism of triplet-triplet absorption (Cherry and Schneider, 1976), and saturation transfer EPR (Hyde and Dalton, 1972; Thomas et al., 1976). These techniques have paved the way for investigations of functional significance of protein mobilities in membranes.

The present study has been undertaken to investigate dynamic aspect of protein-lipid interactions from both sides of the constituents using recombinant membranes of rhodopsin in well-defined phospholipids. Recombinant membrane with purified protein and well-defined phospholipids gives an ideal system to investigate protein-lipid interaction because it simplifies the problem and helps us have a transparent discussion.

The protein rotational mobility was observed by saturation transfer EPR spectroscopy (Hyde and Dalton, 1972; Thomas et al., 1976) of covalently attached spin label. Saturation transfer spectroscopy, a new method of EPR which enables us to detect slower rotational motions with correlation times from  $10^{-7}$  s to  $10^{-3}$  s, is particularly suitable for the study of rotational motions of proteins in membranes. By complexed use of saturation transfer EPR with conventional EPR, we can cover rotational correlation times from  $10^{-10}$  s to  $10^{-3}$  s. Saturation transfer technique, thus, has made spin labeling method unique one among other methods with sensitivity to broadest correlation times. We employed adiabatic rapid passage approach (absorption with phase sensitive detection of second harmonic  $90^\circ$  out-of-phase) to detect transfer of saturation between spin packets caused by reorientation of anisotropic nitroxide radical.

The lipid alkyl chain flexibility was monitored by conventional EPR spectra of the dispersed stearate spin labels. When necessary,

saturation transfer EPR was also used.

Rhodopsin was chosen because of the characteristic function as a photoreceptor protein and also because of a wealth of biophysical and biochemical informations. The rotational and lateral mobilities in intact disc membranes have <sup>(been)</sup> investigated (Cone, 1972; Poo and Cone, 1974) and the results from saturation transfer experiments can be directly compared (Baroin et al., 1977; Kusumi et al., 1978). Preparation of rhodopsin recombinants has been described (Hong and Hubbell, 1972, 1973; van Breugel et al., 1977). Recombinant membranes of rhodopsin has been utilized by many workers to investigate protein-lipid interaction as for lipid mobility (Hong and Hubbell, 1972; Davoust et al., 1979; Kusumi et al., 1979), the decay of Meta I rhodopsin (Applebury et al., 1974; O'Brien et al., 1977), and distribution and mobility of rhodopsin (Chen and Hubbell, 1973; Kusumi et al., 1979).

As the two dimensional matrix, dimyristoylphosphatidylcholine (DMPC) and dielaidoylphosphatidylcholine (DEPC) were employed. These phospholipids have differences in the alkyl chain length ( 4 methylenes) and unsaturation (9-trans). Attention was paid to the phenomena accompanied with lipid phase transition. The phase transition temperatures (12 ° C for DEPC and 22 ° C for DMPC) are not so high that the effect of phase transition on the protein mobility may be studied without thermal denaturation. Recombinants with various rhodopsin/DMPC ratios were also prepared and protein-

protein interaction as well as protein-lipid interaction was studied.

The results showed a strong influence of rhodopsin on the surrounding phospholipid. The protein-lipid interaction modified the phase behavior of phospholipids and affected the protein rotational mobility correspondingly. The importance of protein-protein interaction will also be pointed out.

## Experimental Section

All procedures involving rhodopsin or retinal were carried out under dim red light (19 W bulb, deep red filter No. 3, King Co. Ltd.) or total darkness.

Materials. DMPC and DEPC were synthesized by the reaction of glycerophosphorylcholine with myristic and elaidic acid (Calbiochem), respectively, according to Cubero Robles and van den Berg (1969). The products were pure on silica gel thin layer chromatogram developed with  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}$  (65:35:4 by vols). Fatty acid analysis of the phospholipid by gas-liquid chromatography showed only a trace amount of impurities (less than 1 %). Egg yolk phosphatidylcholine (EYPC) was prepared according to Singleton et al. (1965). Lysophosphatidylcholine was obtained from EYPC by the reaction with phospholipase  $A_2$  (Naja Naja).

Detergents used were as follows: Ammonyx LO (Onyx Chemical), dodecyltrimethylammonium chloride (DTAC) (Tokyo Kasei), cetyltrimethylammonium bromide (Wako Pure Chemical Industries), cholic acid (Wako), and octyl- $\beta$ -glucoside (Sigma). Ammonyx LO and octyl- $\beta$ -glucoside were used without further purification. DTAC was purified by recrystallization from ethanol and ethylacetate twice, cetyltrimethylammonium bromide from methanol and acetone five times, and cholic acid from ethanol and water twice.

$^{14}\text{C}$ -Dodecyltrimethylammonium iodide synthesized by the method of

Hong and Hubbell (1972) was a gift of Dr. T. Maeda of this laboratory.

Concanavalin A-Sepharose 4B was prepared from concanavalin A (Wako) and Sepharose 4B (Pharmacia) by the cyanogen bromide activation procedure (Porath et al., 1967), followed by the addition of 0.5 M monoethanolamine to mask unreacted groups on Sepharose. Concanavalin A-Sepharose 4B purchased from Pharmacia was also used.

11-cis-Retinal was prepared by isomerization of all-trans retinal (Sigma) at 0 ° C for 20 min under sunlight filtered through Hoya glass (Brown and Wald, 1956) and purified by silica gel thin layer chromatography developed with petroleum ether/ethyl ether (80:20 v/v) twice at 0 ° C. High pressure liquid chromatography showed slight contamination of 13-cis-retinal and some degraded products. However, these impurities did not disturb the binding of 11-cis-retinal to opsin.

Tempol and MSL were synthesized by the methods of Briere et al. (1965) and Griffith and McConnell (1966), respectively. Tempo was synthesized from 2, 2, 6, 6-tetramethylpiperidine (Aldrich) and purified by recrystallization. 5- and 16-Nitroxide stearate were synthesized by the method of Waggoner et al. (1969) with slight modification (Ito et al., 1975).

Hemoglobin was prepared as described by Benesch and Benesch (1962) from outdated human red blood cells. BSA (fatty acid free) was



obtained from Armour Laboratories and anti DNP-BSA antisera produced in goat and anti goat-IgG antisera produced in rabbit from Miles. Glutaraldehyde (70 % w/w) from Ladd, N-ethylmaleimide and iodoacetamide from Nakarai Chemicals were used.

#### Preparation and Spin Labeling of Frog Rod Outer Segment

(ROS) Membranes. Frog ROS membranes were isolated from retinas of dark-adapted frog (Rana catesbeiana) by the method of Papermaster and Dreyer (1974). The membranes were used fresh or stored under argon at -80 ° C to protect from oxidation of lipid alkyl chains. The buffer used for the following experiments with frog ROS membranes was 80 mM KCl/40 mM NaCl/15 mM CaCl<sub>2</sub>/10 mM HEPES, pH 7.4 and bubbled <sup>10</sup>thoroughly with nitrogen or argon for the protection against oxidation. The ROS membranes were pretreated with 20 mM iodoacetamide at 0 ° C for 2 h and washed twice by centrifugation. The membranes were then made reacted with MSL at a molar ratio (MSL/rhodopsin) of 100:1 at 0 ° C for 8 h and washed 7 times. When necessary, the spin-labeled membranes were treated with 5 % (w/w) glutaraldehyde at 0 ° C for 2 h and washed once.

Isolation of Bovine Rhodopsin. Isolated bovine retinas were shaken in 34 % (w/w) sucrose in the standard buffer (SB) containing 65 mM NaCl and 10 mM HEPES (pH 6.8). Unless otherwise mentioned, all the experiments for recombinants were carried out in this buffer. ROS membranes were prepared essentially by the method of Papermaster

and Dreyer (1974). Purified rhodopsin was obtained by either of the following three methods.

1) ROS membranes were washed with SB twice by centrifugation and then incubated with 11-cis-retinal for 5 h at room temperature to regenerate rhodopsin from some opsins which had been formed in the slaughter house. The regenerated sample was washed three times with distilled water, lyophilized, and stored under argon at  $-80^{\circ}\text{C}$ . Phospholipid-free rhodopsin was obtained according to van Breugel et al. (1977) with a modification. Lyophilized ROS membranes were dissolved with a medium containing 0.1 M DTAC, 150 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 1 mM  $\text{MnCl}_2$  buffered at pH 6.3 with 0.1 M acetate-sodium acetate. The solubilized rhodopsin was centrifuged at 100,000 g for 1 h. The clear supernatant was applied to concanavalin A-Sepharose 4B column and eluted with the same buffer containing 0.3 M  $\alpha$ -methyl-D-mannoside. The eluted rhodopsin preparation gave an optical purity ( $A_{278}/\Delta A_{498}$ ) of  $1.9 \pm 0.2$ . The molar ratio of phospholipid to 40,000-dalton protein was  $0.5 \pm 0.2$ . The molar extinction coefficient of rhodopsin at 498 nm used was 40,000 and the molecular weight was 40,000. Protein and phospholipid were determined by the method of Lowry et al. (1951) and Bartlett (1959), respectively. On sodium dodecylsulfate polyacrylamide gel electrophoresis (8 % w/v acrylamide), the preparation was pure with a small amount of dimer and trimer bands as Makino et al. (1977) observed.

2) Rhodopsin was partially purified by ammonium-sulfate salting-out described by Makino et al. (1977). The resulting pellet was dissolved in the column-applying solution of method 1) containing 2 % (w/w) cholate instead of 0.1 M DTAC. The following procedures were the same as those in 1). The purified rhodopsin gave an optical purity of around 1.8.

3) ROS membranes were dissolved in a solution containing 2 % (w/w) cholate, 200 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , and 10 mM HEPES (pH 7.4). This solution was centrifuged at 100,000 g for 1 h and the clear supernatant was applied to concanavalin A-Sepharose 4B column. After washing the column with 5 column-volumes of the application medium, the detergent of the washing solution was changed from 2 % cholate to 30 mM octyl- $\beta$ -glucoside and the column was further washed with 10 column-volumes of this solution. Rhodopsin was eluted with the washing solution containing 140 mM  $\alpha$ -methyl-D-mannoside. In the presence of octyl- $\beta$ -glucoside, opsin was eluted, if any, somewhat later compared with rhodopsin (Albert and Litman, 1978). The first several of eluted fractions with optical purity better than 1.9 were collected. The collected solution gave an optical purity of about 1.7 and a molar ratio of phospholipid to 40,000-dalton protein of about 1.0.

Method 1) was used for the preparation of lipid-rich recombinants (rhodopsin:phospholipid = 1:300). Rhodopsin-DMPC recombinants

with various rhodopsin/DMPC ratios were prepared by methods 2) and 3).

Preparation of Recombinant Membrane Vesicles. Recombinant membrane vesicles were prepared virtually by the method of Hong and Hubbell (1972, 1973).

DMPC and DEPC recombinants with rhodopsin/phospholipid ratio of 1/300 (mole ratio) were prepared after rhodopsin was purified by method 1). DMPC or DEPC was solubilized by brief sonication in the column-application medium. The solubilized phospholipid was gently mixed with the purified rhodopsin at a molar ratio of 300:1. Solid DTAC was added to this solution to give a final concentration of 0.3 M and allowed to equilibrate more than 18 h at 0 ° C. DTAC was removed by <sup>exhaustive</sup> dialysis for 5-7 days against 1 mM EDTA in 30 mM acetate buffer at pH 6.3 and then against 1 mM EDTA in 5 mM HEPES at pH 6.8 twice (Applebury et al., 1974). Recombined membrane vesicles were harvested by centrifugation at 16,000 g for 30 min and suspended in 1 mM EDTA at pH 6.8 with 5 mM HEPES. Bio-Beads SM-2 (20-30 mesh, Bio-Rad laboratories) or BSA was added to remove the residual detergent further. A radioactivity measurement using <sup>14</sup>C-dodecyltrimethylammonium iodide indicated that the residual detergent was reduced from one per 21 phospholipids to one per 107 phospholipids by this treatment. Finally, the recombinant vesicles were washed and resuspended in SB. Some rhodopsin was denatured

during the recombination process and the recovery yield was estimated at  $75 \pm 8$  % from the optical purity. Thin layer chromatography showed some degradation of phospholipids to lysophosphatidylcholine, visualized with molybdenum phosphate reagent (Dittmer and Lester, 1964).

Recombinants with varying ratios of rhodopsin/DMPC were prepared after rhodopsin was purified by the method of 2) and 3). DMPC was dispersed in distilled water by vortexing and 8 % (w/w) cholate buffered at pH 7.4 with 30 mM imidazole was added to obtain clear solution. The solubilized DMPC was gently mixed with the purified rhodopsin at a desired ratio. The final concentration of cholate was 2 - 3 %. The mixture was allowed to equilibrate more than 18 h at 0 ° C. Detergents were removed by exhaustive dialysis against solutions containing 30 mM imidazole, 1 mM EDTA, and progressively decreasing concentrations of NaCl (200, 150, 100, 50, 0 mM) at pH 7.4 and then against 1 mM EDTA in 5 mM HEPES at pH 7.4. The recovery yield of rhodopsin was estimated at  $88 \pm 5$  % from optical purity.

Spin Labeling of Recombinant Membranes. Rhodopsin in the recombinant membranes was spin labeled with the maleimide reagent (MSL) at a molar ratio of 1:100 at 0 ° C for 8 h, followed by washings 7 times by centrifugation. When one certain cysteinyl residue was desired to be spin labeled, N-ethylmaleimide was utilized. Before rhodopsin in the recombinant membranes was spin labeled with MSL, the membranes were pretreated with N-ethylmaleimide for 45 min at

0 ° C. The membranes were washed twice by centrifugation and then incubated with 1 mM MSL for 40 h at 0 ° C. Unreacted spin reagent was removed by washings 7 times.

For spin labeling with 5- and 16-nitroxide stearate, the bottom of a test tube was coated with thin film of the label and the membrane suspension was poured to give a molar ratio of the label to the phospholipid from 1:50 to 1:100 for the study of conventional EPR. A ratio of 1:600 was used for saturation transfer EPR. The mixture was kept overnight at 0 ° C and then transferred to another tube, followed by washing once. For Tempo partition experiments, 5 mM Tempo in distilled water was added to the membrane suspension to give a final concentration of 0.4 - 0.6 mM.

Crosslinking of Rhodopsin in Recombinant Membranes. Three ways were adopted for crosslinking of rhodopsin in recombinant membranes.

1) The maleimide spin-labeled recombinants were treated with  $10^4$ -fold excess of 2.5 % (w/w) glutaraldehyde at 0 ° C overnight. Unreacted glutaraldehyde was removed by washing twice by centrifugation. When further crosslinking was desired, BSA was added at 0 ° C to the glutaraldehyde-treated membranes at a final concentration of 5 mg/ml.

2) MSL-rhodopsin was first dinitrophenylated by the reaction with thirty-fold excess of dinitrophenylfluorobenzene at 0 ° C overnight and then washed. Anti DNP-BSA antisera was dialyzed against 150 mM

NaCl at pH 6.8 with 10 mM HEPES before use. The antibody (10 mg) was incubated with about 2 mg of dinitrophenylated rhodopsin in recombinants at 27 ° C for 2 h. The suspension was then centrifuged at 16,000 g for 10 min at 0 ° C and the pellet was resuspended for immediate use of EPR experiments. The rhodopsin-antibody complex was further crosslinked with 2.5 % (w/w) glutaraldehyde.

3) MSL-rhodopsin was first dinitrophenylated by the reaction with 1.2 mM dinitrophenylfluorobenzene at 0 ° C overnight and washed. Anti DNP-BSA antisera produced in goat and anti goat-IgG antisera produced in rabbit was dialyzed against 150 mM NaCl buffered at pH 6.8 with 10 mM HEPES before use. Anti DNP antisera (about 8 mg antibody) was incubated with about 4 mg of dinitrophenylated MSL-rhodopsin at 31 ° C for 2 h. Half of this mixture was used for EPR experiments and the other half was washed three times by centrifugation. The pellet was resuspended and incubated with anti goat-IgG rabbit antisera (about 4 mg antibody) at 31 ° C for 2 h. The mixture was then centrifuged and the pellet was used for EPR experiments.

The mixture was further crosslinked with 5 % glutaraldehyde at 0 ° C overnight after washed twice by centrifugation. Unreacted aldehyde residues were not masked. This mixture was washed three times and incubated with 10 mg of BSA overnight for further cross-linking.

EPR Measurements. Conventional and saturation transfer EPR spectra were measured on a Varian E-12 X-band or JEOL FE-2X spectrometer with variable temperature accessory. Magnetic field modulation at 50 kHz and phase sensitive detection at 100 kHz and 90° out-of-phase was adopted in order to detect passage saturation transfer signal. A "π/2" circuitry, 50 kHz active filter, and a capacitor to resonate cavity coils at 50 kHz were attached to the spectrometer. Rectangular cavity working at  $TE_{102}$  mode was used for Varian E-12. Inside diameter of the sample capillary used was 0.7 mm. Cylindrical cavity working at  $TE_{011}$  mode was used for JEOL FE-2X. Sample capillary with an inside diameter of 0.59 mm was employed.

Saturation transfer spectra are sensitive to the effective microwave field  $H_1$  that depends on the loaded Q-factor. In order to use identical  $H_1$  in experiments under different conditions, we obtained power correction curves (Figure 1A) which were used to readjust the incident microwave power. For this calibration, solid Tempol was attached on the outside of the sample capillary and saturation curve for each sample at each temperature was obtained. Samples used for this purpose were air (vacant capillary), water, glycerol/water (47:53, molar ratio), 40 mg/ml BSA + 75 mM EYPC in SB, and 40 mg/ml BSA + 300 mM EYPC in SB. Even for different samples, two microwave powers on the dial that give the same EPR signal height of Tempol at a certain temperature should give the identical  $H_1$  on the samples.



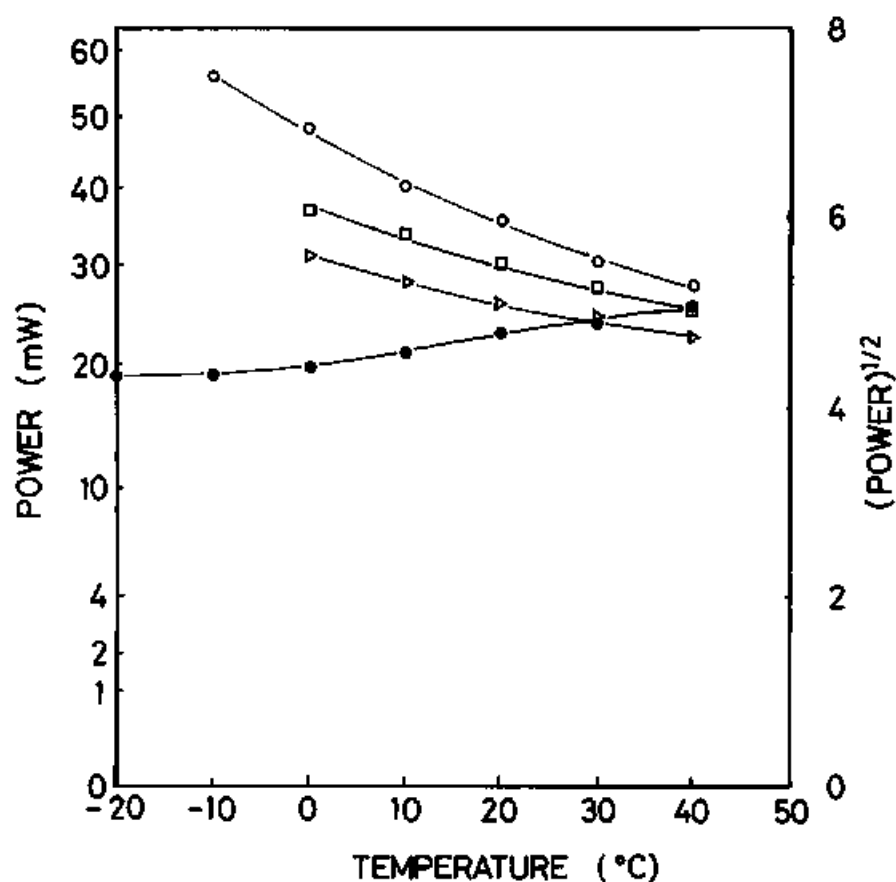


Figure 1A: Power correction curves. Powers on the dial are readjusted to obtain the identical  $H_1$ -field on the sample. These powers shown by the curves correspond to the power on the air sample (vacant sample capillary) of 16 mW at 20 ° C. For details, see text. ● :glycerol/water = 47/53 (molar ratio), ○ :water, □ :40 mg/ml BSA + 75 mM EYPC in SB, ▴ :40 mg/ml BSA + 300 mM EYPC.

Recombinant samples were measured at the same microwave powers as those for water in the first half of this study with Varian E-12 spectrometer. The correlation times obtained there, consequently, gave somewhat underestimate of the value. In the latter half of this work, the humidity (water content) of each recombinant sample was always checked with peak heights of  $\text{MnCl}_2$  in  $\text{MgO}$  that was put simultaneously in the cavity at  $20^\circ\text{C}$ , and we obtained the concentration of EYPC that corresponded to the humidity of the recombinant sample. We determined the working incident microwave power by interpolating in Figure 1A the temperature and corresponding concentration of EYPC. Although the interpolation procedure might not be very accurate, a difference of several mW in incident microwave power did not seem to cause disastrous change in saturation transfer spectra.

The  $H_1$ -field that we worked on in the latter half of this work with JEOL FE-2X corresponded to an incident microwave power of 16 mW for air sample (vacant capillary) at  $20^\circ\text{C}$ , which was the  $P_{1/2}$  value for MSL-rhodopsin (central peak) in ROS membranes at  $20^\circ\text{C}$ .

Estimation of Rotational Correlation Time. The relation between rotational correlation time of a protein and the peak height ratios in the saturation transfer spectrum was obtained by using three kinds of systems: MSL-hemoglobin, MSL-BSA, and 5-nitroxide stearate-BSA. MSL-hemoglobin was prepared by the method of Ohnishi et al.

(1966). BSA was spin labeled by incubating BSA with MSL at a molar ratio of 1:1 at 0 ° C overnight. Unreacted MSL was removed by dialysis. 5-Nitroxide stearate-BSA was prepared by adding BSA solution to a thin film of 5-nitroxide stearate at a molar ratio of 1:1. These samples were mixed with glycerol (glycerol/water = 47/53, molar ratio) and the saturation transfer spectrum was obtained at various temperatures from 40 ° C to -20 ° C. The incident microwave powers used were determined as stated above and shown in Figure 1A. The rotational correlation time was calculated by using 2.9 nm and 3.6 nm as the radius of hemoglobin (McCaley et al., 1972) and BSA (Sober, 1970), respectively, and viscosity of aqueous glycerol (Slie et al., 1966). Graphs relating the peak height ratio to the rotational correlation time were made as shown in Figure 1B and 1C.

As for  $C'/C$  ratio (Figure 1C), the three systems showed a good agreement in all the correlation time range, while  $L'/L$  ratio (Figure 1B) showed some difference between MSL systems and 5-nitroxide stearate system in longer correlation time range. The degree of hydrogen bonding between spin labels and proteins or solvents would be dependent on the kind of spin labels and the local environments of attached spin label (Johnson et al., 1978). This effect should be more prominent in hyperfine interaction than Zeeman interaction, which would make  $L'/L$  ratio sensitive to the kind of spin label and its environment.

The rotational correlation time of MSL-rhodopsin was estimated

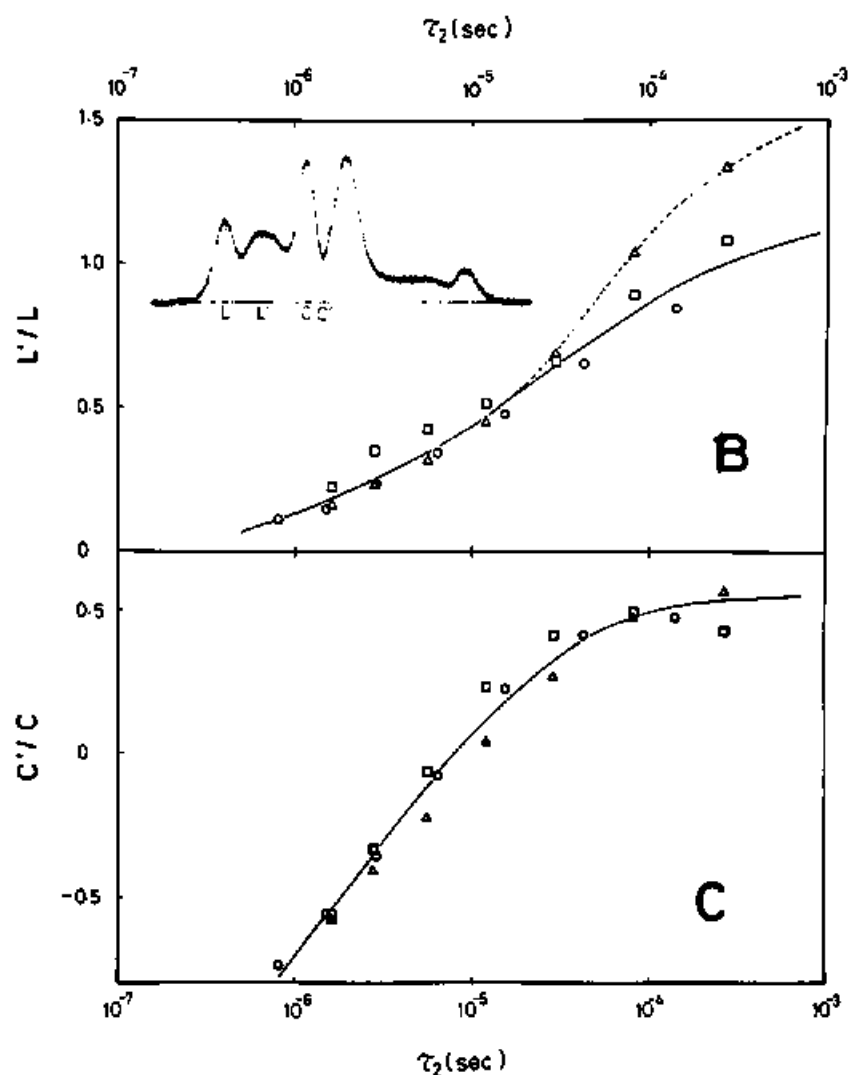


Figure 1B and 1C: Relation between the rotational correlation time  $\tau_2$  and the peak height ratios of saturation transfer spectrum. 1B:  $L'/L$  vs.  $\tau_2$ , 1C:  $C'/C$  vs.  $\tau_2$ . The second harmonic  $90^\circ$  out-of-phase absorption was measured at various temperatures in glycerol/water (47:53, molar ratio) for MSL-hemoglobin  $\circ$ , MSL-BSA  $\square$ , and 5-nitroxide stearate-BSA  $\triangle$ . Microwave powers shown in Figure 1A were used. Field modulation amplitude (peak-to-peak) was 5.6 gauss.

from  $L'/L$  ratio, which is more sensitive to slower motion than  $C'/C$  ratio (full line in Figure 1B). Preliminary evaluation of the motion of 5- and 16-nitroxide stearate by saturation transfer EPR was obtained from  $L'/L$  ratio (dotted line in Figure 1B) and  $C'/C$  ratio. The motion of these molecules must be anisotropic (Kusumi et al., 1978, 1979), so the estimate was adopted as a convenient measure for the rotational mobility of these molecules. The estimated value should, however, give a reasonable order of magnitude (Cone, 1972; Baroin et al., 1977; Kusumi et al., 1978).

Conventional EPR Measurements. Conventional EPR experiments were carried out with subsaturating microwave power (usually 5 mW) on the dial. The frequency of field modulation was either 10 kHz or 50 kHz or 100 kHz. Modulation width (peak-to-peak amplitude) used was from 1.4 to 2 gauss.

Light Irradiation. Light from a 1 kW projector lamp was used with filters: Toshiba VR-62 or VO-58 to obtain light with wavelengths longer than 620 nm or 580 nm, respectively, for frog ROS experiments and Toshiba VR-60 and IRP-70 to obtain light around wavelengths between 600 - 700 nm. Recombinant membranes were illuminated intermittently 10 x (10 s irradiation and 20 s dark) at temperatures above phospholipid phase transitions.

## Results

In this chapter we first describe the rotational motion of frog rhodopsin in ROS membranes and then protein-lipid interaction in recombinant membranes.

### I The Rotational Motion of Rhodopsin in ROS Membranes

We started this work with the study of rotational motion of rhodopsin in frog ROS membranes because the rotational relaxation time had been investigated by transient photodichroism (Cone, 1972) and the results can be directly compared each other.

A conventional EPR spectrum of the spin-labeled membrane is shown in Figure 2A. The overall splitting value was  $65.7 \pm 0.20$  gauss at  $20^\circ\text{C}$ . A trace amount of narrow component (see arrow) can be seen in addition to the rigid component. The narrow component increased when the molar ratio of spin label reagent to rhodopsin was decreased. Since it perturbs estimation of the correlation time from the saturation transfer spectrum, a higher ratio was employed for the spin labeling. The pretreatment with iodoacetamide also decreased the narrow component. The trace narrow component did not practically affect the saturation transfer spectrum. The number of spin labels attached to one mole of rhodopsin was 2.2, in agreement with the previously published data (de Grip et al., 1973; Delmelle and Virmaux, 1977). The spin label signal gradually decreased owing to reduction of the nitroxide moiety (see Figure 3A).

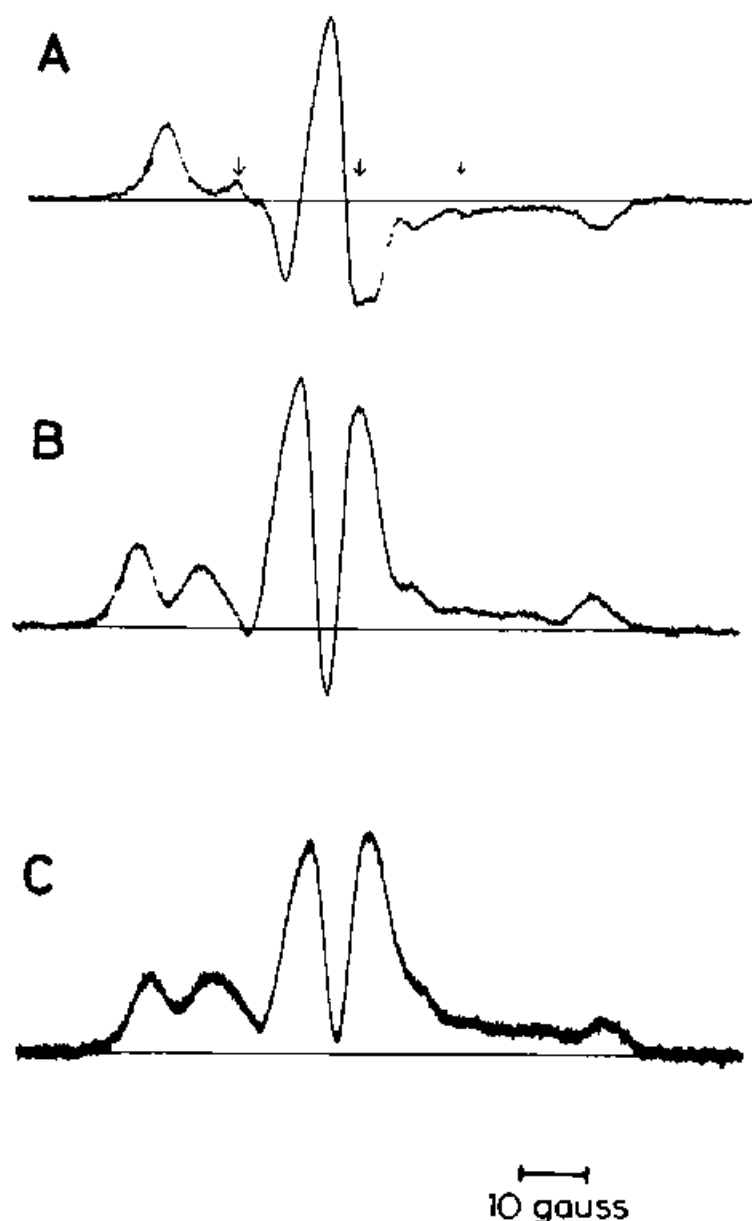


Figure 2: EPR spectrum of spin-labeled ROS membranes at 20 °C. (A) Conventional measurement: first harmonic in-phase absorption spectrum at an incident microwave power of 1 mW and with field modulation (10 kHz) amplitude (peak-to-peak) of 2 gauss. (B) Saturation transfer spectrum: second harmonic out-of-phase absorption with an incident microwave power of 63 mW and field modulation (50 kHz) amplitude of 4.2 gauss. (C) Saturation transfer spectrum of glutaraldehyde-fixed membranes.

The saturation transfer spectrum of the spin-labeled ROS membrane is shown in Figure 2B. The peak height ratios  $L'/L$ ,  $C'/C$ , and  $H'/H$  were measured for more than 7 spectra, yielding  $0.75 \pm 0.015$ ,  $-0.27 \pm 0.029$ , and  $0.49 \pm 0.026$ , respectively. The  $C'/C$  ratio was smaller immediately after the spin labeling, increased gradually with time, and reached a stable value of  $-0.27$  after a few hours (see Figure 3B). The rotational correlation time was obtained by referring to Figure 3C as 20, 3.6, and 13  $\mu\text{s}$  from the  $L'/L$ ,  $C'/C$  (stable value), and  $H'/H$ , respectively. The difference in the values is probably caused by the use of model spectra obtained with BSA. This protein has a nearly spherical shape and the rotational motion must be isotropic. The result, therefore, suggests anisotropic motion of rhodopsin in the membrane. Generally, the saturation transfer spectrum should be greatly dependent on the orientation of the spin label with respect to the protein rotational axis. When the spin label y axis coincides with the rotational axis, for example, the correlation time would be underestimated. This tendency is more pronounced in the central part of the spectrum. For correct estimation of the correlation time we need to have suitable model spectra or computer-simulated spectra for the anisotropic rotation.

The glutaraldehyde-fixed membranes gave the saturation transfer spectrum as shown in Figure 2C. All the peak height ratios increased markedly, indicating reduction of the rotational motion.



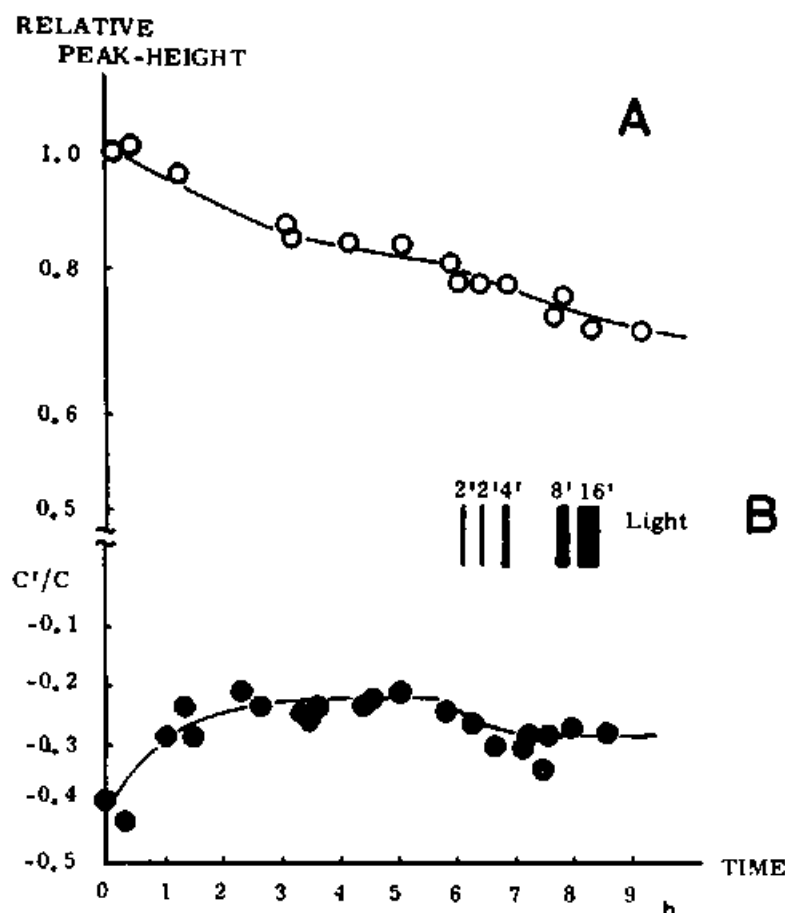


Figure 3: (A) Decay of the EPR signal of spin-labeled ROS membranes. The low field peak height of the conventional EPR spectrum was plotted against time at 20 °C. (B) Dependence of the peak height ratio  $C'/C$  of the saturation transfer spectrum on the storage time in the dark (before 5.8 h) and on intermittent illumination using a cut-off filter at 620 nm at 20 °C. The inset shows the time of illumination in minutes. (C) Dependence on the rotational correlation time  $\tau_2$  of the peak height ratios of the saturation transfer spectrum. 5-Nitroxide stearate-BSA in glycerol/water (47:53, molar ratio) was measured at various temperatures on a Varian E-12. These graphs were utilized to obtain  $\tau_2$  values of rhodopsin in Figures 2 and 9.

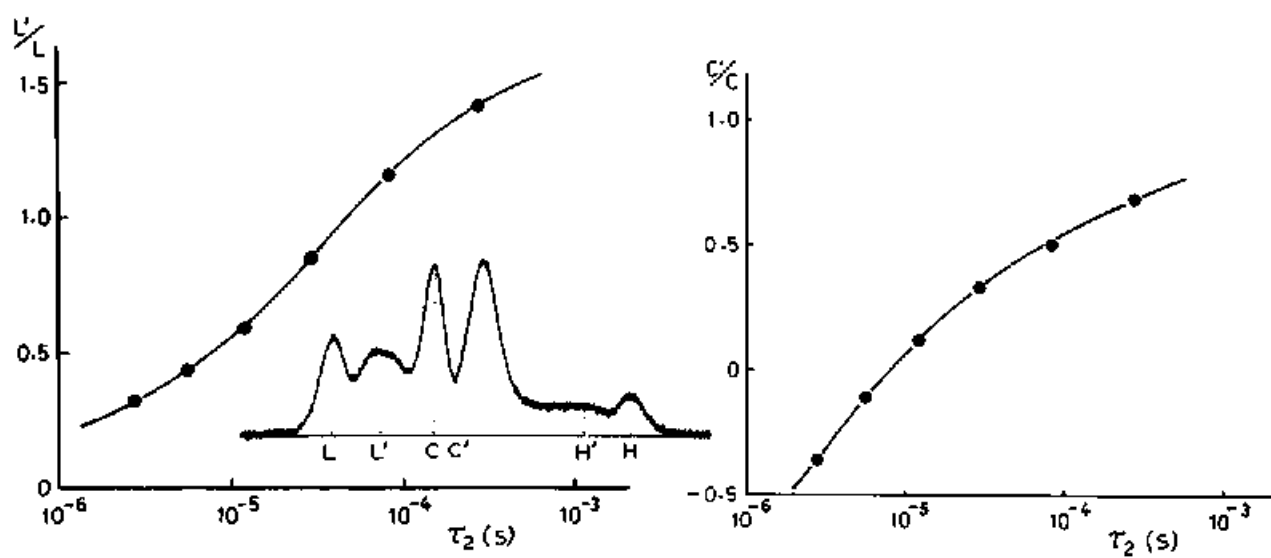


Figure 3C

The  $L'/L$ ,  $C'/C$ , and  $H'/H$  were  $1.00 \pm 0.011$ ,  $0.051 \pm 0.020$ , and  $0.80 \pm 0.011$ , which led to the correlation time of 45, 9.6, and 45  $\mu$ s, respectively. The conventional EPR spectrum (not shown) was affected only slightly. The overall splitting value was  $66.4 \pm 0.25$  gauss at 20 °C. A sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated exhaustive crosslinking of rhodopsin molecules.

Cone (1972) has obtained 20  $\mu$ s at 20 °C for the rotational relaxation time of rhodopsin in frog retina. The value is similar to that obtained in the present study. After glutaraldehyde treatment, Cone has observed no decay of the dichroic ratio even after 10 s at 6 °C. The present result also indicated a restriction of rotational motion on glutaraldehyde treatment. However, the correlation time was much less than 10 s. The difference may be reasonably explained by considering the difference in the principle of the measurement. The saturation transfer spectrum would be affected by rotations with the angle large enough to jump over a spin packet. A few degrees of oscillatory rotations would be enough to modify the spectrum.

The spin-labeled membrane was bleached stepwise by illumination with light at wavelengths longer than 620 nm. The effect on the saturation transfer spectrum was slight but definite. The  $C'/C$  value decreased gradually as photobleaching went on and became  $-0.31 \pm 0.027$  on complete bleaching (Figure 3B). The  $L'/L$  value was almost unchanged,  $0.77 \pm 0.016$ . Further irradiation with light at wavelengths longer than

580 nm for 10 min caused no change in saturation transfer spectra. Whether this change is due to change in the rotational motion or in conformational change is to be studied. The photobleaching did not affect the conventional EPR spectrum.

## II Protein-Lipid Interaction in Rhodopsin Recombinant Membranes

In this section we first report the effect of rhodopsin incorporation on phospholipids. The incorporated rhodopsin gave largest effect on phospholipid molecules around its phase transition, and a large effect in the fluid state, while it gave small influence in the solid state. The decreased mobility of lipids affected rotational motion of rhodopsin on the other hand. Latter half of this section describes the mobility of rhodopsin in recombinant membranes.

Effect of Incorporation of Rhodopsin on Phospholipids. The phase behavior and motion of the hydrophobic chain of phospholipid were monitored by using 5- and 16-nitroxide stearate. The temperature was always lowered after it was brought to the highest temperature. Figure 4 shows the temperature dependence of overall splitting value ( $2T_{1/2}$ ) of the conventional EPR spectra of 5-nitroxide stearate dispersed in lipid-rich recombinant (1:300) with DMPC and DEPC. They were prepared by using DTAC as a detergent. The splitting value changed abruptly near the phospholipid phase transition temperature. Incorporation of rhodopsin caused a broadening of the transition

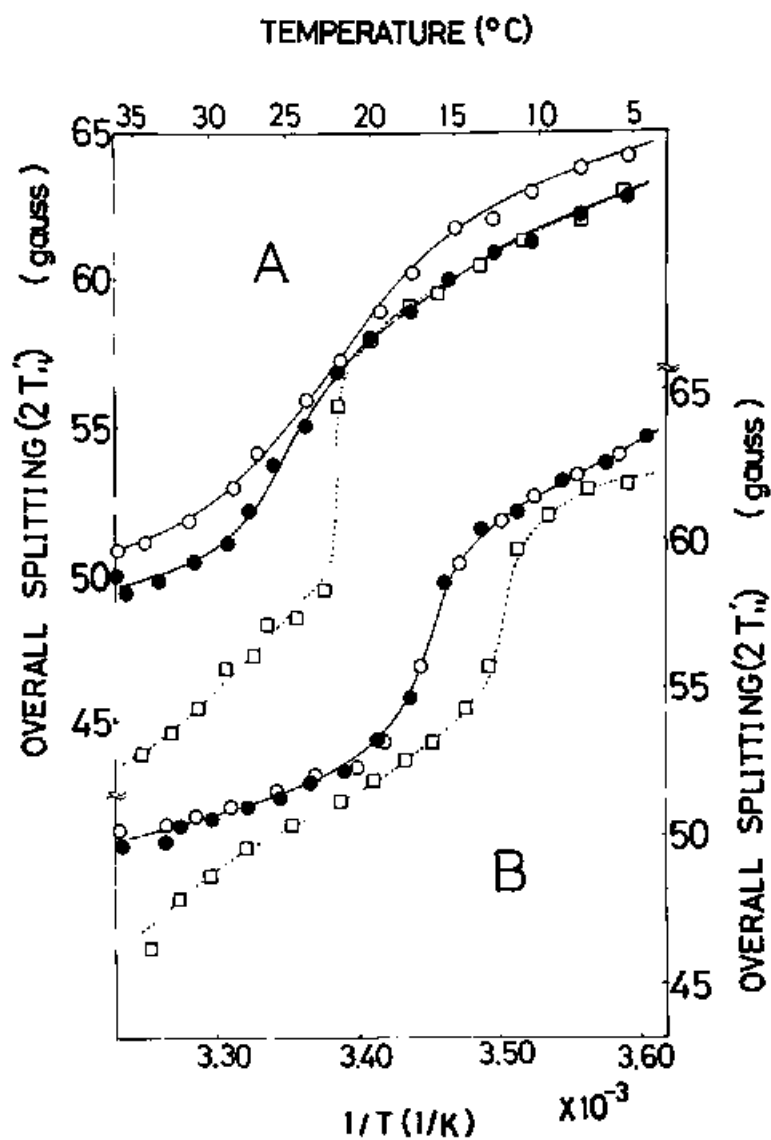


Figure 4: Temperature dependence of the overall splitting value for 5-nitroxide stearate in DMPC (A) and DEPC (B) recombinants. The dotted lines are for the corresponding phospholipid bilayer membranes. Unbleached ● and bleached ○ recombinants measured in the dark (lipid:rhodopsin = 300 :1). □ Phospholipid dispersion by Vortex mixer.

and shifting of its midpoint to higher temperature.

The incorporated rhodopsin gave a large effect on phospholipid molecules in the fluid state, while it gave little influence in the solid state. The overall splitting value for DMPC recombinant was almost indistinguishable below the phase transition temperature from that for pure DMPC bilayer membranes prepared by vortexing, but it was much larger above the temperature (Figure 4A). This represents a marked slowing down of the molecular motion of 5-nitroxide stearate alkyl chain in the fluid state. Effect of rhodopsin on DEPC was less pronounced (Figure 4B). The overall splitting value for the recombinant was larger than that for DEPC bilayer in the fluid state but the difference was smaller than that for DMPC. Tempo partition experiments gave analogous results to those obtained by the stearate.

The observed effects of rhodopsin on the phase behavior do not appear to be artefact due to residual detergent. Recombinant preparation containing different amounts of DTAC (one DTAC per 21 and 107 phospholipids) gave the same results with each other. Effect of detergent on pure phospholipid membranes was also checked. Phase behavior of DMPC was not disturbed by DTAC at least 5 mole %. DEPC was more sensitive than DMPC and, while 1 mole % of DTAC had no effect, 5 mole % of the detergent broadened and shifted the transition to lower temperature.

Formation of some lysophosphatidylcholine was observed

when recombinant membranes were formed by using DTAC. The effect of lysophosphatidylcholine on DMPC bilayer is shown in Figure 5. Lysophosphatidylcholine did not cause a change in temperature characteristics of the phase transition up to 20 mole % of total lipid. The overall splitting value was increased in the fluid state by lysophosphatidylcholine, while it was decreased in the solid state. The change was, however, very small when the amount of lysophosphatidylcholine was 10 mole % of total lipid. No formation of lyso-form was observed when cholic acid or octyl- $\beta$ -glucoside was used as a detergent. Recombinants with various rhodopsin/DMPC ratio were prepared by these detergents.

Figure 6A shows the overall splitting values ( $2T_{11}$ ) of conventional EPR spectra of 5-nitroxide stearate dispersed in DMPC recombinants with various ratios of rhodopsin/DMPC, plotted against temperature. The increase in rhodopsin/DMPC ratio caused more broadening of the transition and more shifting of its midpoint to higher temperatures. DMPC molecules in the fluid state were much more affected by the incorporated rhodopsin than those in the solid state. The motion of 5-nitroxide stearate was shown to become more slowed down as more rhodopsin was incorporated in the membrane. These features of the effect of rhodopsin incorporation are manifested more clearly by plotting  $\Delta(2T_{11}) = 2T_{11}(\text{Recombinant}) - 2T_{11}(\text{DMPC})$  against temperature, where  $2T_{11}(\text{Recombinant})$  and  $2T_{11}(\text{DMPC})$  represent the overall split-

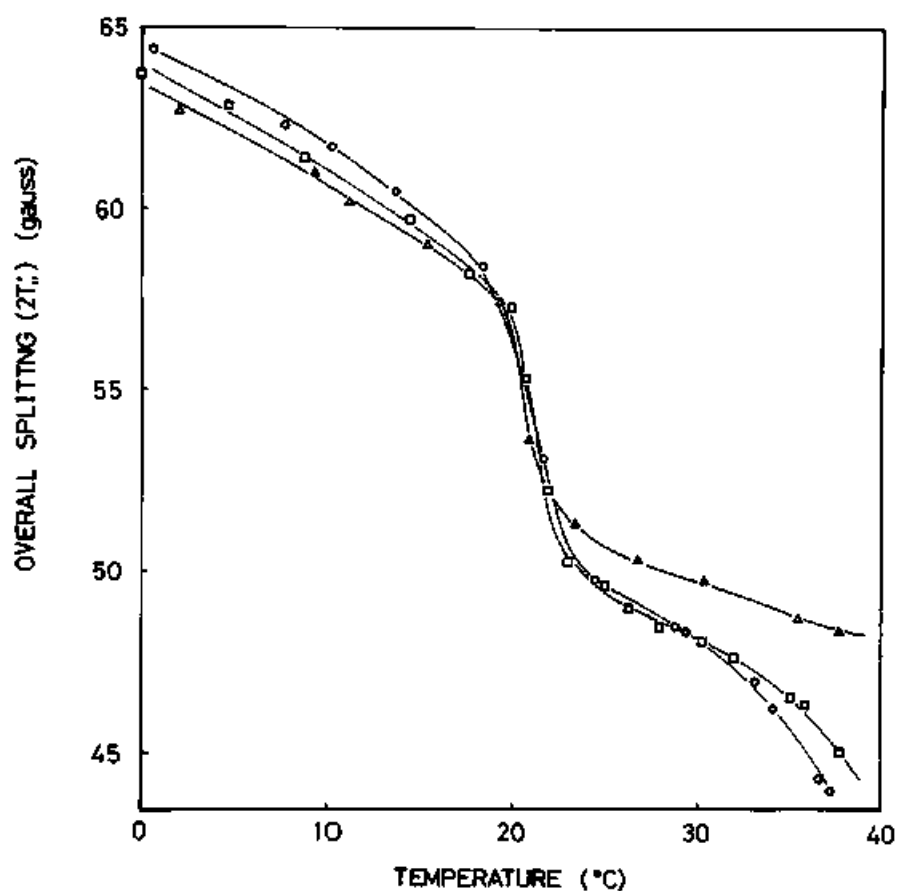


Figure 5: The effect of lysophosphatidylcholine on the phase behavior of DMPC. Phospholipids were dispersed by Vortex mixing. ○ : pure DMPC, □ : DMPC:lysophosphatidylcholine= 9:1 (molar ratio), Δ : DMPC:lysophosphatidylcholine=8 : 2 (molar ratio).



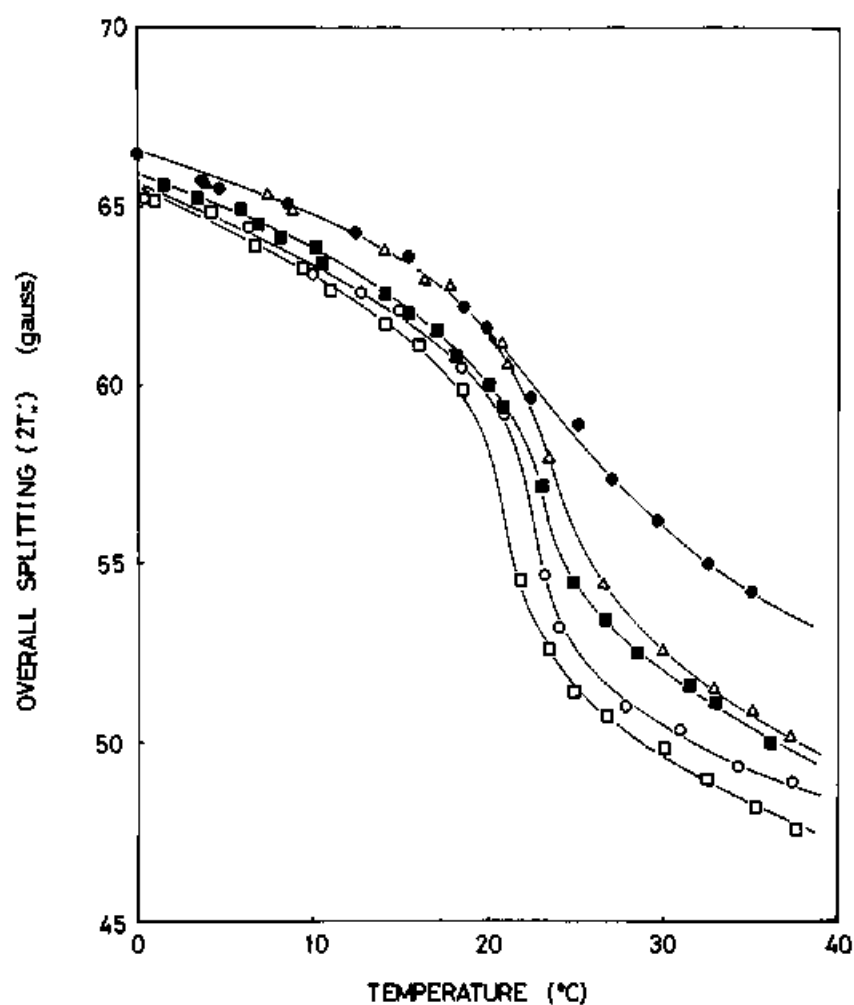


Figure 6A: Temperature dependence of the overall splitting value for 5-nitroxide stearate in DMPC recombinants with various rhodopsin/DMPC ratio. The ratios (molar ratios) are 1 : 22 ● , 1 : 43 ▲ , 1 : 90 ■ , and 1 : 153 ○ . □ : DMPC membranes prepared by cholate dialysis.

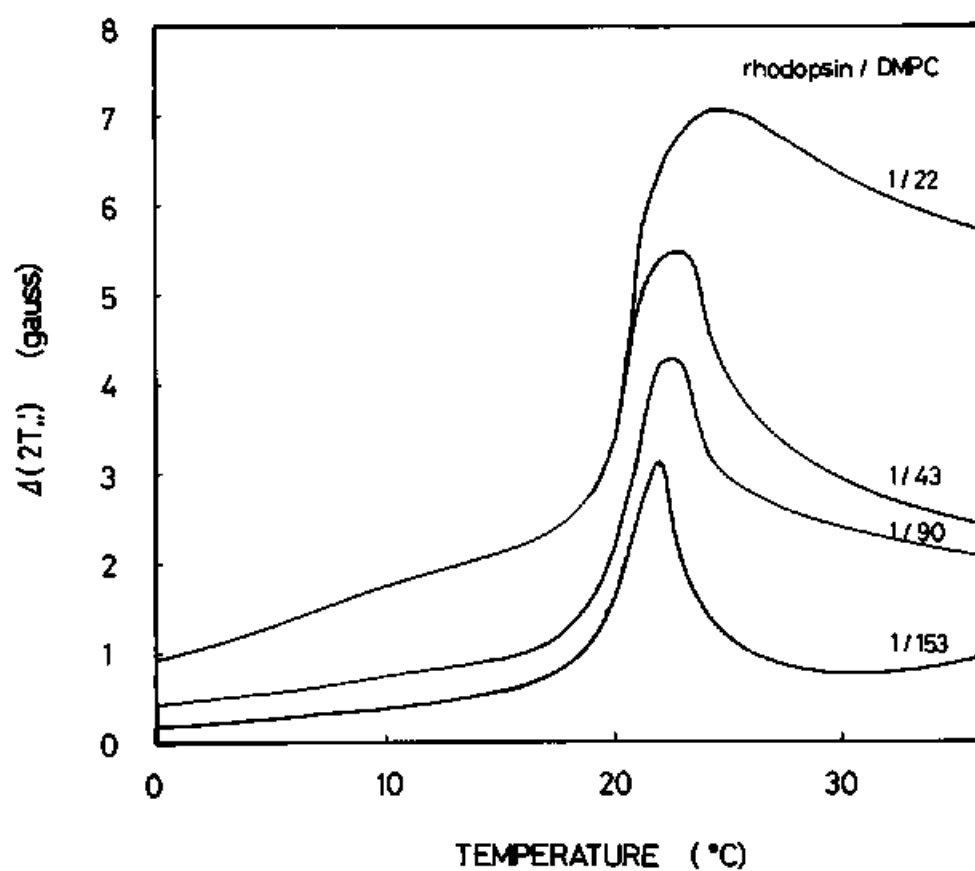
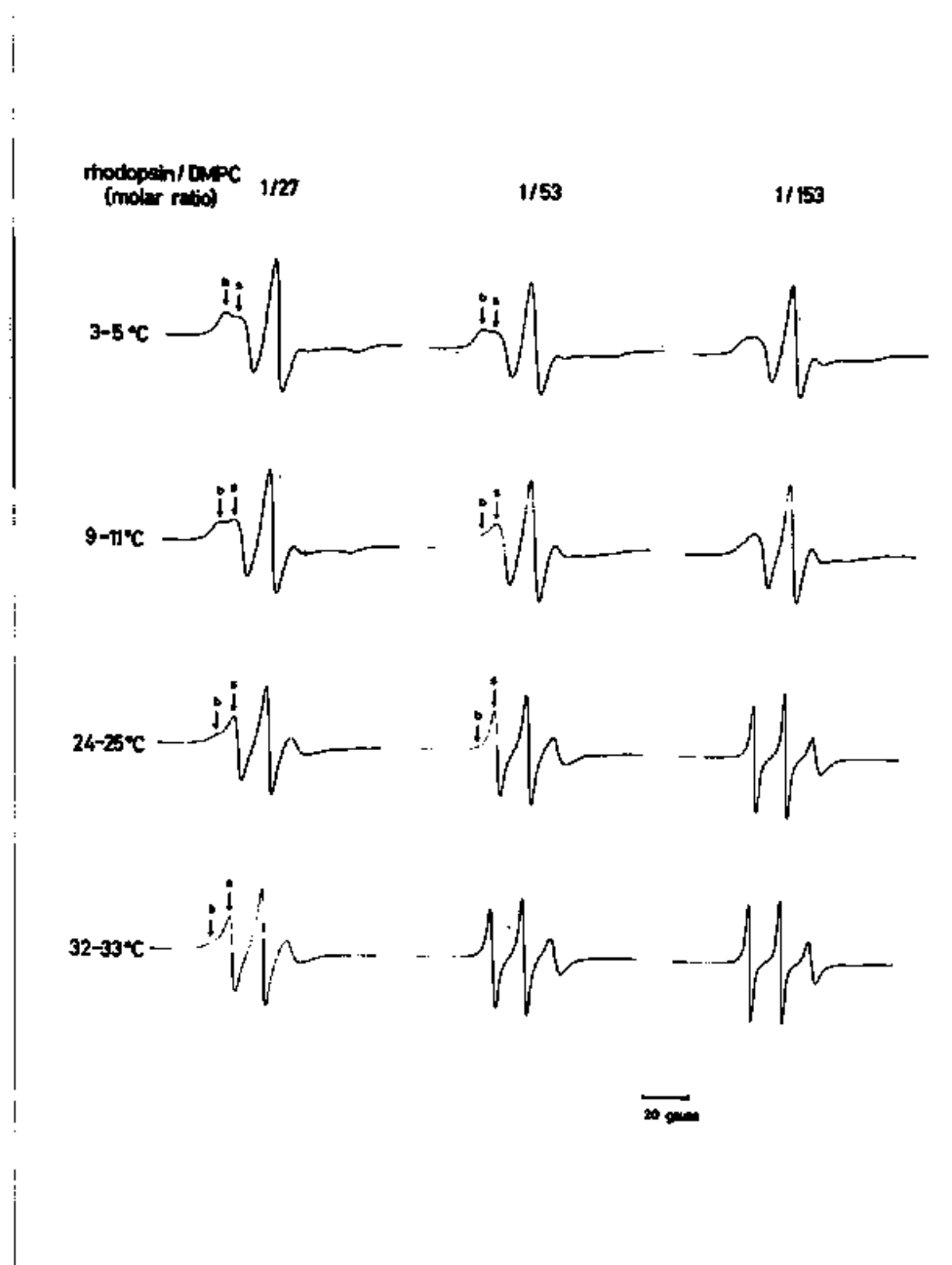


Figure 6B: Temperature dependence of  $\Delta(2T_{II}^{\uparrow}) = 2T_{II}^{\uparrow}(\text{Recombinant}) - 2T_{II}^{\uparrow}(\text{DMPC})$ . Subtractions were made in Figure 6A.

ting values for recombinant membranes and DMPC membranes prepared by dialysis, respectively (Figure 6B).

Figure 7 shows the conventional EPR spectra of 16-nitroxide stearate. The spectra indicated more slowing down of motion of 16-nitroxide stearate when the concentration of protein in the lipid was increased above the phase transition temperature. Even in the lipid-rich recombinant with rhodopsin/DMPC ratio of 1/153, spectral change was observed to indicate some kind of decrease in the mobility of 16-nitroxide stearate (full and dotted lines in Figure 7). Two spectral components, broad peak (b in Figure 7) and sharp one (s in Figure 7), which have been assigned to protein-bound and bulk lipid, were seen in the lipid-poor recombinant with rhodopsin/DMPC ratio of 1/27. When the temperature was lowered, the peak height of sharp component decreased greatly. The decrease resulted in more pronounced two spectral components in recombinants with rhodopsin/DMPC ratios of 1/27 and 1/53 below the phase transition temperature. This result is in good accord of the data observed by Hesketh et al. (1976) in  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -dependent ATPase from sarcoplasmic reticulum complexed with dipalmitoylphosphatidylcholine by using the same spin label. The overall splitting value of broad component was larger than that of DMPC bilayer in solid phase, indicating slower motion of 16-nitroxide stearate of this component than the spin label in DMPC bilayer below the phase transition temperature.



**Figure 7: Conventional EPR spectra of 16-nitroxide stearate in DMPC recombinants with different rhodopsin/DMPC ratio at temperatures both above and below the phase transition temperature. When two spectral components are seen, they are shown by arrows: b for broad component, s for sharp component.**

### Rotational Motion of Rhodopsin in Recombinant Membranes.

Conventional and saturation transfer EPR spectra of MSL-rhodopsin in recombinants are shown in Figure 8A and 8B, respectively. No weakly immobilized component can be seen in the conventional spectrum. The rotational correlation time of rhodopsin was evaluated from the peak height ratio of  $L'/L$  in saturation transfer spectra.

Figure 9 shows temperature dependence of the rotational correlation time of MSL-rhodopsin in lipid-rich recombinants with DMPC and DEPC (rhodopsin/DMPC = 1/300, molar ratio). Membranes were spin labeled without pretreatment. A discontinuous change was observed near the corresponding phospholipid phase transition. The change was broad and occurred on the higher temperature side compared with the <sup>pure</sup> phospholipid transition. The characteristic temperatures of the profile are tabulated in Table 1. The temperatures agree well with those of the same recombinant observed from the lipid side. When the curves for DMPC- and DEPC-recombinants were superposed (full and dotted line in Figure 9), the curves coincide with each other in the solid state. In the fluid state, however, the rotational correlation time for DEPC recombinant was larger than that for DMPC recombinant.

The temperature dependence followed almost straight lines both above and below the phase transition temperature (Figure 9). The slope was steeper in the solid state than in the fluid state, indicating higher barrier for the rotational motion in the solid state.

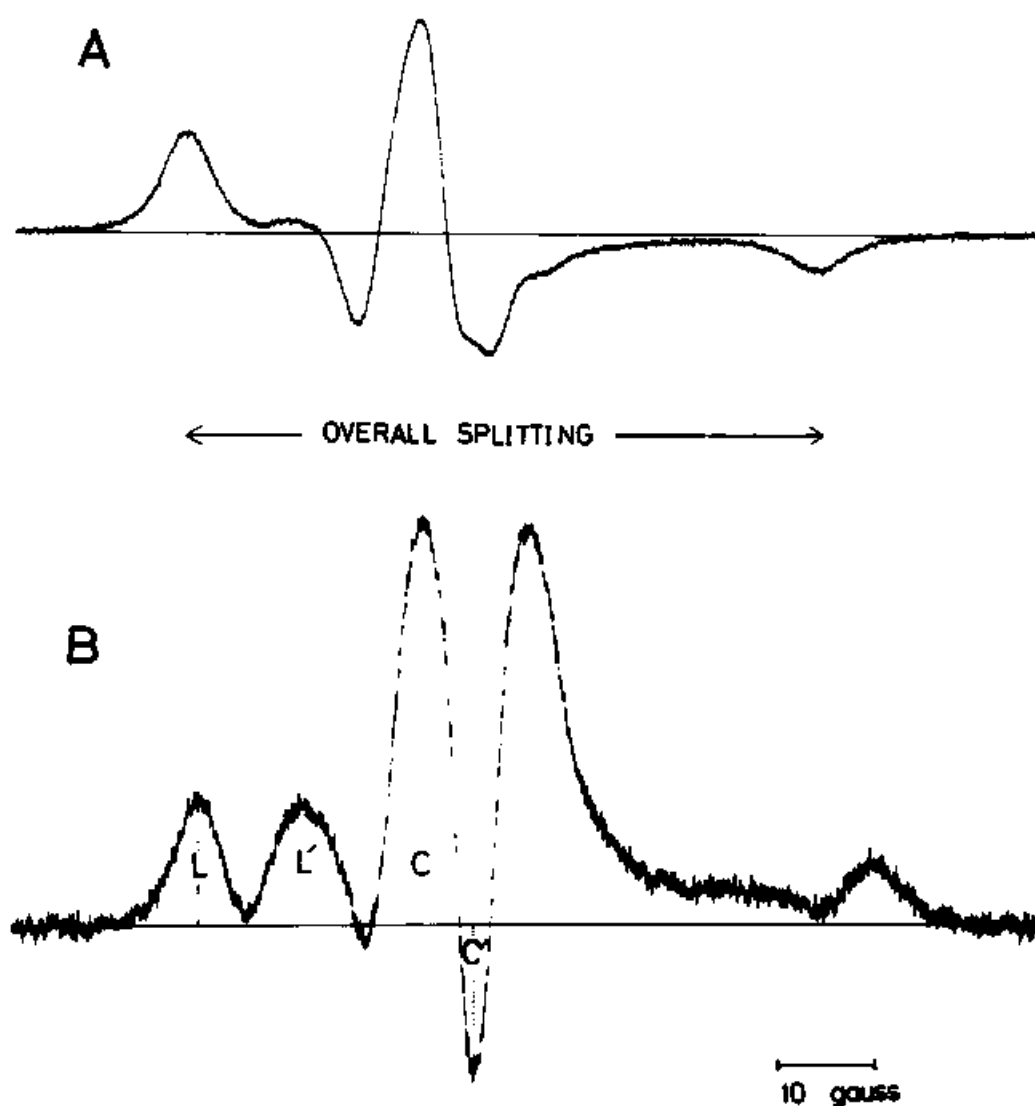


Figure 8: Conventional (A) and saturation transfer (B) EPR spectra of maleimide spin label attached to rhodopsin in DMPC recombinaant. Temperature, 10.4 °C. Modulation width: 2 gauss (A) and 4.2 gauss (B). Modulation frequency: 10 kHz (A) and 50 kHz (B). Incident microwave power: 10 mW (A) and 70.5 mW (B) on the dial. Both spectra were measured on a Varian E-12.

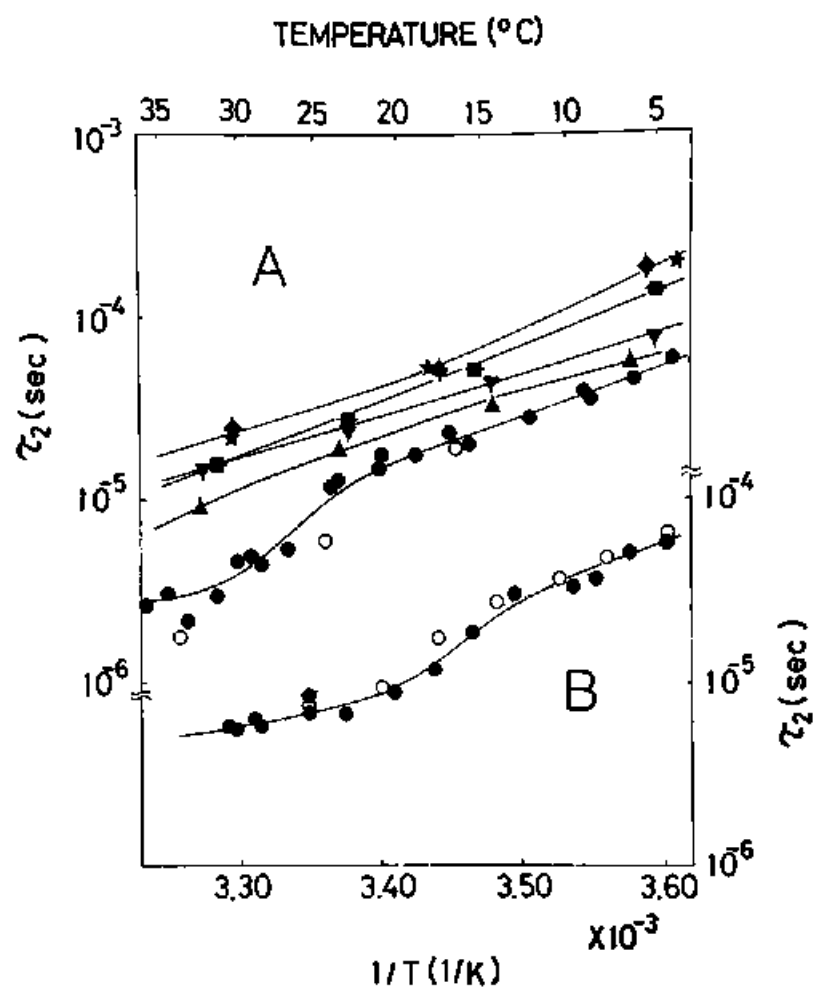


Figure 9: Apparent rotational correlation time of rhodopsin in DMPC (A) and DEPC (B) recombinants. Unbleached ● and bleached ○ recombinants measured in the dark. The dotted curve in (B) is a reproduction for DMPC recombinant. Effect of various treatments of DMPC recombinant on the correlation time is included in (A): ▲ glutaraldehyde, ▼ glutaraldehyde and BSA, ■ anti DNP antisera after dinitrophenylation, ◆ glutaraldehyde treatment of ■, BSA treatment of ◆.

TABLE 1: Characteristic Temperatures for Rhodopsin Recombinant in DMPC and DEPC. Lipid alkyl chain flexibility<sup>a</sup> and rotational correlation time of rhodopsin<sup>b</sup> were measured as a function of temperature and the inflection point at higher ( $T_h$ ) and lower ( $T_l$ ) temperatures and the mid<sup>^</sup>point temperature ( $T_m$ ) were read.

$$\Delta T = T_h - T_l.$$

	$T_h$	$T_m$	$T_l$	$\Delta T$
DMPC	23.1°	22.2°	18.8°	4.3°
DMPC recombinant, lipid <sup>a</sup>	31.0°	25.0°	18.3°	12.2°
DMPC recombinant, rhodopsin <sup>b</sup>	32.9°	27.2°	20.0°	12.9°
DEPC	13.2°	12.1°	8.4°	4.8°
DEPC recombinant, lipid <sup>a</sup>	23.8°	17.0°	12.1°	11.7°
DEPC recombinant, rhodopsin <sup>b</sup>	22.7°	16.6°	10.0°	12.7°



Photobleaching of these recombinants did not affect the rotational correlation time appreciably (Figure 9).

Recombinants were subjected to various treatments and the effect on the rotational correlation time was examined (Figure 9A). This was done in order to check whether the motion being observed approximated rotational motion of rhodopsin as a whole. Firstly, recombinants were incubated with large excess of glutaraldehyde. This treatment increased the rotational correlation time significantly. The increase was larger in the fluid phase (about 3 fold). BSA was added to the glutaraldehyde-treated recombinants and made reacted for 1 h at 0 ° C. This resulted in further restriction of the rotational motion. Simple addition of BSA to recombinants gave no effect on the motion.

Glutaraldehyde treatment restricted the rotational motion but the restriction was not complete. Glutaraldehyde molecules exist in the form of  $\alpha, \beta$ -unsaturated aldehyde polymers with different degrees of polymerization and generate Schiff base linkage with amino groups when allowed to react with proteins (Peters and Richards, 1977). The crossbridges of glutaraldehyde polymers are thus long and flexible so that they may allow some oscillatory rotation of the protein in a limited angle. This may explain the residual mobility after glutaraldehyde treatment, in the same way as that for intact ROS membranes. Further inhibition of the rotational motion by BSA may be due to linking

between amino groups of BSA and the residual aldehydes in the glutaraldehyde crossbridges, causing further crosslinking.

As a different approach, recombinants were first dinitrophenylated and then incubated with anti DNP antisera. The rotational correlation time was greatly increased (3 to 5 fold). Dinitrophenylation alone did not affect the motion. Glutaraldehyde treatment of the antibody-bound recombinants caused further immobilization. Further addition of BSA gave no effect.

Effect on Rotational Motion of Rhodopsin of Increasing Rhodopsin/DMPC Ratio. Specific labeling of rhodopsin at a certain cysteinyl residue was tried. Rhodopsin was usually spin labeled with MSL after it was pretreated with N-ethylmaleimide. The MSL/40,000-dalton protein ratio obtained was 1.1. According to Fung and Hubbell (1978), the attached MSL should be located on the Rh<sub>27</sub> segment by their notation, carbohydrate-carrying segment. Another experiment was tried in which rhodopsin was first incubated with MSL at 0 ° C for 45 min and then treated with N-ethylmaleimide. The MSL/40,000-dalton protein ratio was 1.2. MSL should attach to Rh<sub>12</sub> segment, retinal-carrying segment (Fung and Hubbell, 1978).

The difference in obtained correlation time is seen in Figure 10. The estimated correlation times were almost the same for rhodopsin labeled on Rh<sub>27</sub> segment and on Rh<sub>12</sub> segment ( □ and ■ in Figure 10, respectively). When both of the two sites were labeled, smaller cor-

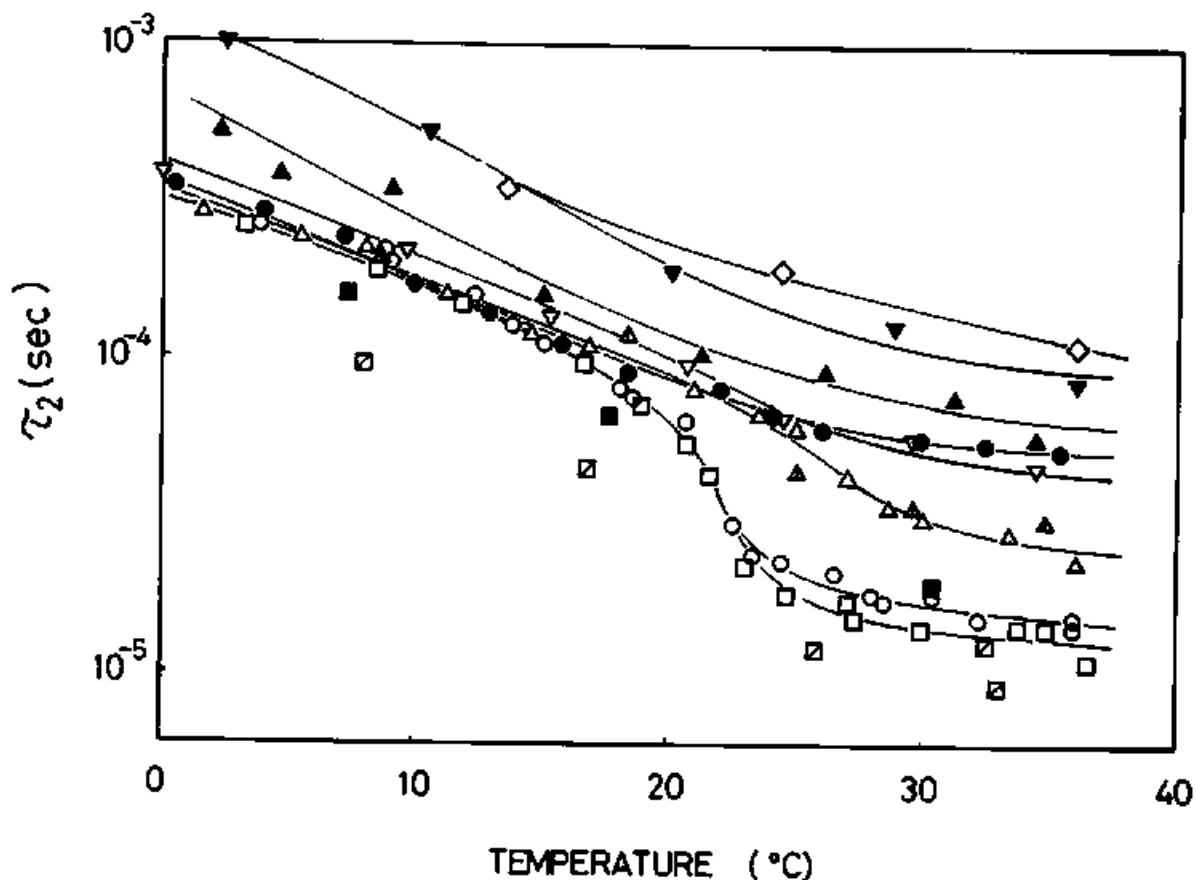


Figure 10: Apparent rotational correlation time of rhodopsin in DMPC recombinants with various rhodopsin/DMPC ratio. Recombinants with rhodopsin/DMPC ratio of 1/240 were spin-labeled with MSL by different procedures;  $\boxtimes$  : two cysteinyl residues on rhodopsin were spin labeled,  $\blacksquare$  : MSL on Rh<sub>12</sub> segment,  $\square$  : MSL on Rh<sub>27</sub> segment. Others are spin labeled on Rh<sub>27</sub> segment: Rhodopsin/DMPC = 1/240  $\square$  , 1/153  $\circ$  , 1/43  $\triangle$  , and 1/22  $\bullet$  . Effect of successive cross-linking treatments of DMPC recombinant (rhodopsin/DMPC = 1/43, molar ratio) on the correlation time of rhodopsin is also included;  $\triangle$  : MSL-rhodopsin without dinitrophenylation + anti DNP antisera (control),  $\nabla$  : dinitrophenylated MSL-rhodopsin + anti DNP antisera produced in goat,  $\blacktriangle$  :  $\nabla$  + anti goat-IgG antisera produced in rabbit,  $\blacktriangledown$  : glutaraldehyde treatment of  $\blacktriangle$  ,  $\diamond$  : addition of BSA to  $\blacktriangledown$  .

relation times were obtained (  $\square$  in Figure 10). This result would be interpreted in terms of spin-spin interaction in a rhodopsin molecule. The effect of spin-spin interaction on saturation transfer was examined with DMPC bilayers containing various amount of 5-nitroxide stearate. The result is listed in Table 2, showing similar tendency as Wilkerson et al. (1978). The dipolar interaction of spin labels attached to rhodopsin has already been reported by Delmelle and Virmaux (1977). The estimated values in Figure 9 were, therefore, somewhat underestimated.

The overall splitting values of conventional EPR spectra were the same (66.1 gauss) at room temperature irrespective of the labeling method in the range of experimental error.

The temperature dependence of rotational correlation time (Figure 10, MSL on Rh<sub>27</sub> segment) was analogous to that of overall splitting value of 5-nitroxide stearate (Figure 6A). The increase in rhodopsin/DMPC ratio induced more broad change in the correlation time at temperatures corresponding lipid phase change. The effect of increasing amount of protein in recombinants was very pronounced in the fluid phase of DMPC, while it was almost indistinguishable in the solid state.

Recombinants were subjected to successive crosslinking treatments and the effect on the rotational correlation time was examined again in lipid-poor recombinant (rhodopsin/DMPC = 1/43, molar ratio) (Figure 10). Recombinants were first dinitrophenylated and then incubated with anti DNP antisera produced in goat. The rotational

TABLE 2: The Effect of Spin-Spin Interaction on Saturation Transfer Spectra. 5-Nitroxide-stearate and DMPC were mixed with various ratios and dispersed by vortexing in SB. The concentration of DMPC was 0.1 M.

5-nitroxide-stearate / DMPC	7 ° C		25 - 27 ° C
	L'/L	C'/C	C'/C
1/30	0.36	-0.76	-1.47
1/100	0.55	-0.47	-1.42
1/300	0.66	-0.38	1.33
1/600	0.67	-0.33	1.18
1/1200	0.69	-0.28	1.20

correlation time increased twice in the fluid state of DMPC, while the increase was smaller in the solid state. Simple addition of the antisera to recombinants gave no effect on the motion. Secondly, anti goat-IgG antisera produced in rabbit was added to the anti DNP antibody (goat) -bound recombinants. The mobility of rhodopsin was reduced at all temperatures. Thirdly, this complex was treated with 5 % (w/w) glutaraldehyde, which further decreased the observed mobility. Further addition of BSA increased the rotational correlation time by crosslinking unreacted aldehyde residues of glutaraldehyde on proteins. These results would be best understood as indicating that the observed mobility of MSL chiefly represented rotational motion of rhodopsin as a whole in recombinant membranes.

Conventional EPR Spectra of Spin-labeled Rhodopsin. The overall splitting value of the conventional spectra of MSL-rhodopsin was plotted as a function of temperature (Figure 11). Only the results from lipid-rich recombinants (1:300) is shown and to be compared with Figure 9. The splitting value for DMPC recombinants changed rather monotonously with temperature, while that for DEPC recombinant showed some discontinuous change near the phase transition temperature. Various treatments on DMPC recombinants did not give effects in a definite way on the spectra, in contrast to the effect on saturation transfer spectra.

Photobleaching of the recombinants gave some definite effect

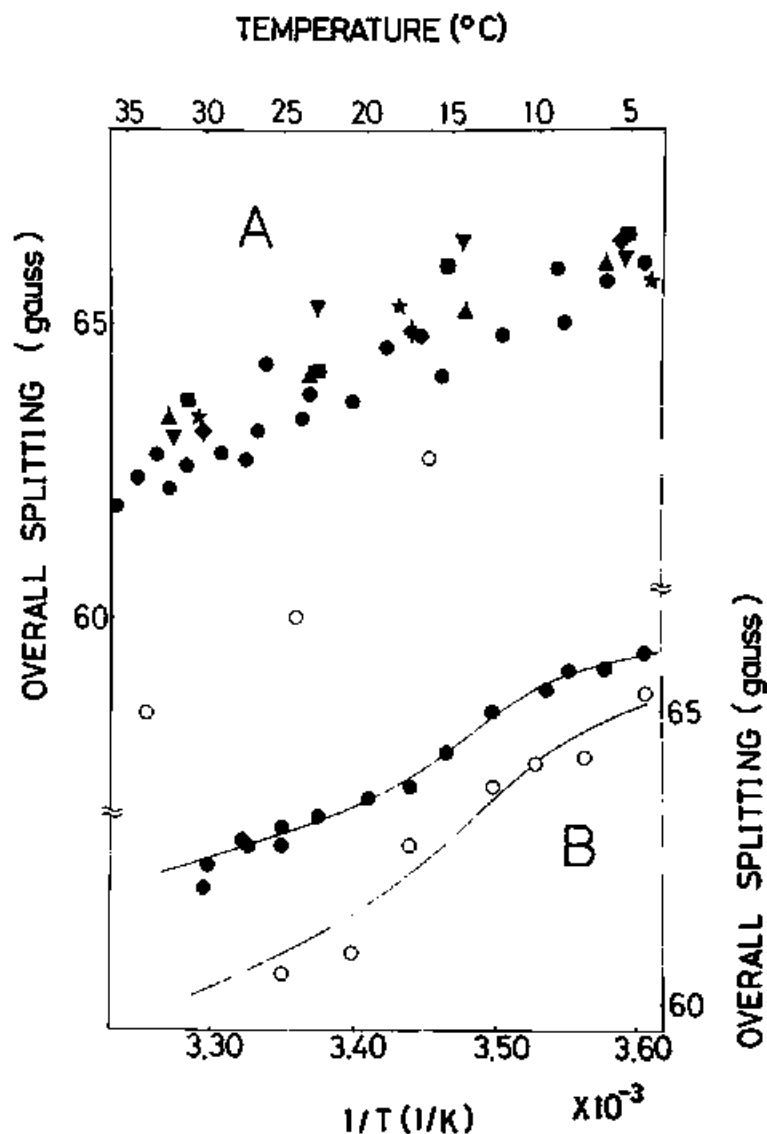


Figure 11: The overall splitting value of conventional EPR spectra of MSL-rhodopsin in DMPC (A) and DEPC (B) recombinants. Unbleached ● and bleached ○ recombinants measured in the dark. Molar ratio of rhodopsin/phospholipid was 1:300. Effect of various treatments of DMPC recombinant on the splitting value is included in (A). The symbols used are the same as in Figure 9.

on the overall splitting value. The splitting value was decreased in both kinds of recombinants, showing some mobilization of the spin label attached to rhodopsin cysteinyl residues. Whether this change is related to protein conformational change of physiological significance remains to be studied.



## Discussion

The incorporation of rhodopsin caused slowing down of the molecular motion of 5- and 16-nitroxide stearate and rhodopsin itself. The slowing down of the alkyl chain motion was most pronounced around phase transition temperature (Figure 6B). The disturbance caused by proteins propagated to longer distances around the temperature, in good agreement with theoretical studies (Marčelja, 1976; Schröder, 1977; Owicki et al., 1978). A large lateral compressibility of phospholipid bilayer, ie, longer correlation length of order parameter, around the temperature would explain the long-range influence of proteins.

The effect of rhodopsin on phospholipids was large in the fluid state but small in the solid state. The latter can be explained by sequestering of rhodopsin from phospholipid phase on crystallization. Freeze-fracture electron micrograph of DMPC recombinant showed a complete phase separation into protein-rich region and crystalline phospholipid (Chen and Hubbell, 1973). This explains the small difference between the lipid flexibility in DMPC recombinant and that in pure DMPC bilayer below the phase transition temperature. The sequestering of rhodopsin also explains why rhodopsin mobility did not depend on rhodopsin/DMPC ratio in the solid phase of the membrane. It is to be noticed that rhodopsin kept some mobility in its

cluster below the phase transition temperature.

In the fluid state, rhodopsins are dispersed into the fluid medium as observed by electron microscopy (Chen and Hubbell, 1973) and exert their influence on the surrounding phospholipids. Effect of rhodopsin on DEPC was less pronounced. The rigidifying effect in the fluid state was smaller than that in DMPC. Electron micrographs indicated that rhodopsin are not fully dispersed at 20 ° C, well above the phase transition temperature, although at still higher temperature, 37 ° C, they are randomly dispersed in DEPC medium (Chen and Hubbell, 1973).

Presence of boundary lipids in a layer adjacent to integral protein surface has been demonstrated in cytochrome oxidase (Jost et al., 1973) and  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -dependent ATPase from sarcoplasmic reticulum (Nakamura and Ohnishi, 1975) when protein/lipid ratio was fairly high. The boundary lipid alkyl chains interact with the hydrophobic protein surface via van der Waals forces and are immobilized in the conventional EPR time scale,  $10^{-7}$  s. When protein hydrophobic surface extended longer than the fluid lipid alkyl chains, the boundary lipids would take more extended chain conformations to cover the surface. This effect would propagate to phospholipids in the next layer and make their conformations more extended or ordered than those in bulk fluid phase. The protein would thus be able to extend

its effect on the outer layer phospholipids and make them more rigid. This may be another reason for the smaller effect of rhodopsin on longer DEPC than that on DMPC, and is consistent with our recent observation of small effect on dipalmitoylphosphatidylcholine in the fluid state.

The incorporation of more rhodopsin caused more slowing down of the alkyl chain motion of 5- and 16-nitroxide stearate. On the other hand, deuterium magnetic resonance (DMR) study by Kang et al. (1979) showed that the introduction of cytochrome oxidase into phosphatidylcholine bilayer produced smaller quadrupole splittings of the deuterium at the terminal methyl groups of alkyl chains. This result implies some kind of increased disorder at alkyl chain terminal. In order for a slower motion (EPR results) to cause smaller quadrupole splittings (DMR results), the alkyl chain motion must become larger in amplitude as was suggested by them (Kang et al, 1979). This conclusion leads us to depict some generalizable features of the effect of protein on phospholipids. The incorporated protein exerts its influence on the surrounding phospholipids via attractive forces that are stronger than those of phospholipid-phospholipid interaction in fluid state as an average (Pink and Chapman, 1979). The other effect is to produce defect of packing in phospholipid bilayer (Chapman et al., 1979). The former effect would cause the slower alkyl chain

motion, while the latter would give larger available area for an alkyl chain, allowing the chain terminal to swing broader.

We would also like to point out the difference of the sensitivity in motional modes to incorporation of proteins. The alkyl chain motion in membranes must be very anisotropic. We can think of two fundamental motional modes: trans-gauche isomerization in an alkyl chain and rotation of lipid around its symmetry axis. It is likely that incorporated proteins affect trans-gauche isomerization more greatly than the rotation of lipids around its symmetry axis on the basis of EPR results and DMR data. Saturation transfer EPR can report the degree of the anisotropy of the motion (Kusumi et al., 1978; Marsh, 1978). In our preliminary study, the correlation times estimated from  $L'/L$  ratio of 5-nitroxide stearate showed about an order of magnitude longer value than that from  $C'/C$  ratio in solid state recombinants. Moreover,  $L'/L$  ratio was relatively insensitive to the change in temperature below the phase transition temperature.  $C'/C$  ratio changed greatly in this temperature range. These results indicate that the dominant motion at C-5 position of stearate was the rotation around the symmetry axis of the molecule rather than trans-gauche isomerization in the solid state recombinants. The overall splitting value of conventional EPR spectra has marginal sensitivity to this kind of motional mode. DMR of deuterated terminal methyl

groups is sensitive to the rotation around the symmetry axis of the molecule, along with fast rotation around the terminal C-C bond, producing small quadrupole splittings.

Above the phase transition temperature, DMR spectra of 6'-labeled DMPC showed that the quadrupole splittings were almost insensitive to the incorporation of cytochrome oxidase, although broadening of the lines was observed (Kang et al., 1979). Overall splittings of conventional EPR spectra of 5-nitroxide stearate greatly increased by incorporated rhodopsin, showing slowing down of the alkyl chain motion with respect to the symmetry axis of its molecular motion. All these data are consistent with the interpretation that incorporation of proteins slows down trans-gauche isomerization (swinging motion of alkyl chains) more effectively than rotation around the symmetry axis.

Conventional EPR spectra of 16-nitroxide stearate showed two components in the lipid-poor recombinants with rhodopsin/DMPC ratio of 1/27 (Figure 7). The number of phospholipid in direct contact with rhodopsin was calculated to be 25-29 (Stubbs et al., 1976; Watts et al., 1978) from the sizes of rhodopsin and phospholipid. If every rhodopsin molecule was surrounded by one layer of phospholipids, the spectra of 16-nitroxide stearate should give one component at a rhodopsin/DMPC ratio of 1/27. The existence of two components

would reflect overlapping of protein annuli in the lipid-poor recombinant. This result suggests that some fractions of DMPC were in multiple contacts with proteins, in agreement with the view presented by Chapman et al.(1979).

The rotational motion of rhodopsin itself was also affected by the incorporated rhodopsin, in good accord with data by Cherry et al. (1977) and Baroin et al. (1979). A good agreement was observed between the temperature characteristics of recombinants as measured by the protein mobility and by the lipid flexibility (Table 1). Another good correlation was obtained when the protein mobility was replotted against the lipid flexibility (Figure 12). It is shown that the rotational correlation time is determined by the lipid fluidity, irrespective of the kinds of phospholipids, in the solid and mid-transition regions if rhodopsin/phospholipid ratio is the same. An approximate linear relationship in those regions indicates that the activation barrier for the rotational motion is related to available area for each lipid. In the fluid state, however, the plots for DMPC deviated from the linearity and became different from that for DEPC recombinant (lower left in Figure 12). This may reflect change in the aggregation state of rhodopsins in DMPC and remaining aggregation in DEPC on the phase transition.

The slowing down of the motion of rhodopsin caused by other

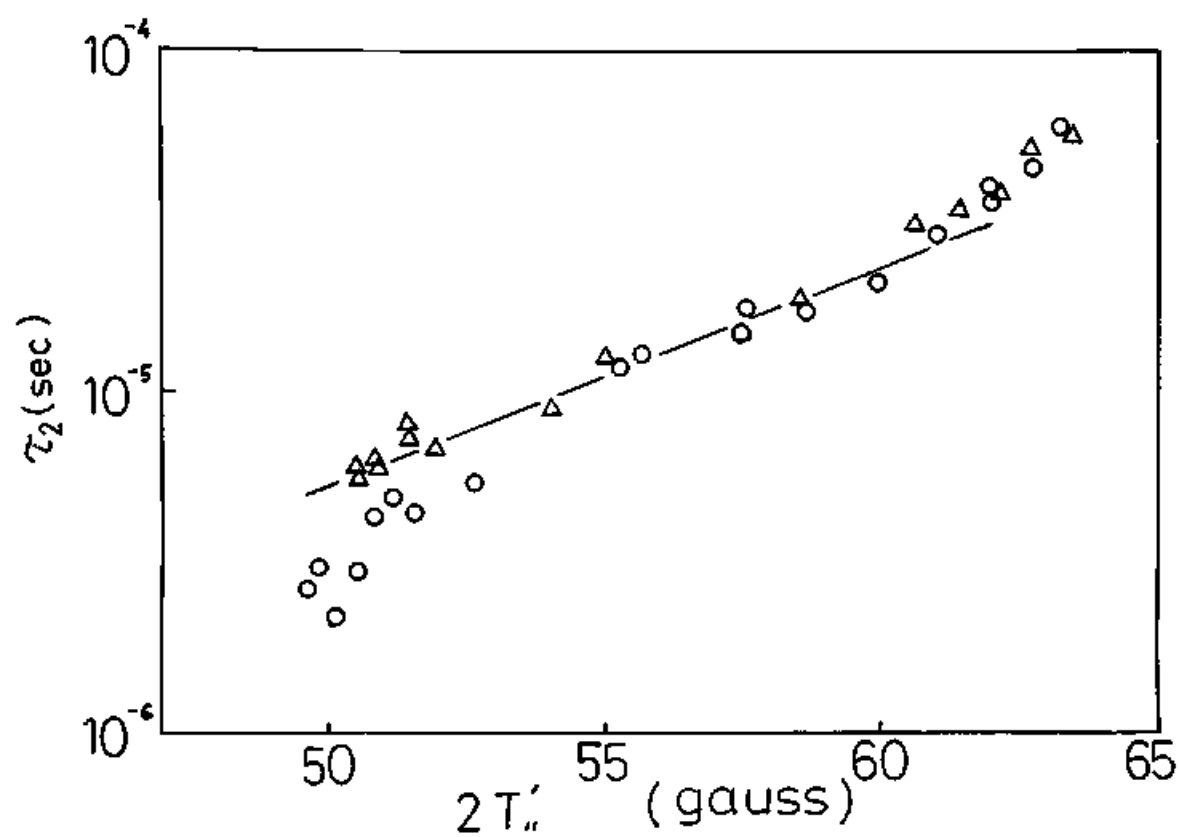


Figure 12: Rotational correlation time of spin-labeled rhodopsin vs. lipid alkyl chain flexibility in DMPC (○) and DEPC (Δ) recombinant membranes. Data replotted from Figures 4 and 9.

rhodopsins in the fluid state may show that rhodopsin "feels" its environment to be more rigid when hydrophobic chains of phospholipids move slower but broader. Another possible reason for the slowing down would be protein-protein interaction. Protein-protein interaction would manifest itself when annuli of proteins come to overlap, even if there is no direct contact between proteins (Schröder, 1977). The overlapping of protein annuli would take place more easily when protein/phospholipid ratio increases, as was described above.



## Concluding Remarks

Saturation transfer spectra<sup>were</sup> shown to be very effective to study the rotational mobility of rhodopsin.

Rhodopsin incorporated into membranes caused slowing down of molecular motion of 5- and 16-nitroxide stearate and rhodopsin itself. A good correlation was observed between temperature profiles as measured by rotational mobility of rhodopsin and alkyl chain flexibility. The slowing down of alkyl chain motion was most pronounced around the phase transition temperature. It is suggested that alkyl chain mobility of trans-gauche isomerization was more affected by incorporated protein than the rotation around the normal axis of the bilayer membrane. Importance of relative dimension of the protein hydrophobic surface to the fluid lipid chain length was pointed out for determining the type and range of the propagating effect of protein incorporation.

Segregation of rhodopsin was a key factor to interpret phenomena observed below the phase transition temperature. This suggests the importance of protein-protein interaction in the membrane. The interaction between proteins that share annular lipids would cause significant slowing down of protein motion.

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